PROBLEMS IN THE ARBOVIROLOGY OF THE BONNEVILLE BASIN, UTAH

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1972

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Pertinent to the results of a serological survey in 1961 of West Central Utah in which there appeared both complement fixing (CF) and serum neutralizing (N) antibodies against three arboviruses, (1) an arboviral survey was initiated by the Army Ecology and Epidemiology Group at Dugway. A subsequent report by Thorpe et al (2) included evidence of CF antibodies against the viruses of Western, Eastern, St. Louis and Venezuelan encephalitides (WE, EE, SLE and VE respectively); confirmation of findings was performed under Dr. T. H. Work, 1963, then at the Virology Section, Communicable Disease Center (3), Atlanta, Georgia. Since the observations were made in and adjacent to the Proving Ground at Dugway, Utah, it became the purpose of these studies to inquire into the stability or the transiency of these antibodies to the area, particularly those of the VE virus, known to be of tropical ecology.

At the time of program inception in 1963, emphasis was placed upon a serological survey of native rodents, rabbits and livestock of the Great Salt Lake Basin and its surrounding areas. In 1965 emphasis was turned toward mosquito isolation studies as a more efficient approach to an inquiry into the arboviral ecology within this area of the Bonneville Basin. Since that time the Army Ecology and Epidemiology group has isolated the Modoc virus (MOD) from the Deer mouse, Peromyscus maniculatus (3), and also the following mosquito-borne viruses: In 1965 WE, California encephalitis (CE) and La Crosse (LAC) viruses (4); in 1966-1967 CE and Cache Valley (CV) viruses (5), and also CE, CV, Jamestown Canyon (JC) and Hart Park-Like (HPL) viruses (6); in 1968 CE, WE, Trivitattus (TVT) and

* The experimental animals used in these studies were housed, fed, and cared for in a humane manner, and such care was supervised by a competent biologist in accordance with principles of laboratory-animal care established by the National Society for Medical Research.

Bunyamwerra Group viruses (7). Host mosquito species included Aedes dorsalis, A. nigromaculis, Culiseta inornata, Anopheles freeborni and psorophora signipennis. Referable to mosquito pool size the isolation of mixed viruses from pools of 100 were not uncommon. This was shown in 1965 wherein twenty-six pools yielded isolates of both CE and WE viruses; in each instance these were A. dorsalis mosquitoes(7).

The geography of the Bonneville Basin has been described by Pack (8). Most of western Utah lies within this Basin, an area of approximately 54,000 square miles, which is the easterly of the two major depressions of the Great Basin. The floor of the Bonneville Basin contains a large number of local depressions, the lowest and largest of which is situated somewhat north of the center and is occupied, in part, by the Great Salt Lake. The Basin is bounded on the east by the Wasatch and Uinta mountains and the High Plateaus of southern Utah within which numerous peaks rise above 12,000 feet elevation. At the south west and north the limiting uplands are of lower elevation. A large number of north-south mountains and valleys modify the floor of the Basin. Annual precipitation ranges from five inches on the western desert floor to 35 inches in the higher mountains of the eastern periphery.

Incident to the requirements for a more valid interpretation of findings associated with the serological survey and alsc of the arbovirus isolation studies, the following investigations were pursued: (1) Experimental clarificetion of sero-diagnostic titer relationships in native mammals, (2) Comparative VE virus infection studies in native rodents and (3) Experimental viral interference studies in suckling mice as pertains to the sensitivity and specificity of diagnostic tests.

EXPERIMENTAL

Diagnostic Titer Relationships in Native Mammals

Table 1 provides informative data showing the relationship of haemagglutination inhibition (HI) titers to plaque neutralizing (N-pr) titers in chick embryo cell culture. The procedures included the microtiter technique for the HI antibody determinations using sucrose acetone extraction of serum and the suckling mouse neutralization tests as outlined by Hammon and Work (1964) (9). Plaque neutralization testing was accomplished on plaque assay plates in which viruses were assayed on primary chick-embryo cell culture media (CEC) under Agar overlay as described by Dolana (1968) (10).

The results in Table 1 show that those HI titers which are relatable to the four significant plaque neutralizations are shown as 1:320, 1:80, 1:160, 1:160. The problem area thus lies with the failure of the remaining HI titers of 1:80 to be indicative of N antibody also.

385

SMART

TABLE 1

HAEMAGGLUTINATION INHIBITION (HI) AND PLAQUE NEUTRALIZATION REACTIONS OF MAMMAL SERA COLLECTED IN THE BONNEVILLE BASIN 1966 - 1967

Serum Description	Species	HI Titer*		Ave. Plaque Count	
		WEE	VEE	WEE	VEE
Control	Domestic Rabbit	-	-	100	200
661532	Desert Wood Rat	20	±80	TNC†	TNC†
661625	Cottontail	neg**	±80	TNC	TNC
661720	Jack Rabbit	±40	320	TNC†	0††
661816	Deer Mouse	neg	80	48	1.5††
662019	Striped Skunk	±160	20	TNC	TNC
670734	Pinyon Mouse	10	160	TNC	0††
670749	Deer Mouse	10	160	TNC	0††
670773	" "	neg	±80	TNC	101
670783	" "	20	±80	TNC	115

* Reciprocal of end point dilution
** Less than 1:10
† No reduction in the number of plaques expected
†† Definite reduction in the number of plaques expected

From these data one must view with suspicion any titer of 1:80 or less in interpreting the HI test, although one would have missed the single N positive animal whose HI titer was 1:80.

In an attempt to provide an answer to this type of problem experimental VE virus infection studies in the coyote, Canis latrans merriam, were extended to elucidate comparative titer relationships (11). In this study fifty-four coyote pups 1-2 months of age and 18 pups 6-7 months old were divided into groups of 6 each and inoculated with 10-fold serial dilutions of VE virus. Sera collected after inoculations were tested for viremia and antibody levels. Coyotes

were bled prior to inoculation and at 1, 2, 3, 4 and 6 weeks after inoculation. Seven of the 1-2 month old coyotes were selected from surviving animals and bled for sera at 7, 21, 31 and 41 weeks. The sera were tested for HI reactions with VE, WE and EE antigens. The results for coyote number 4372 are shown in Table 2. Maximal CF titers (1:64) were reached in two weeks at which time a neutralization index of 3.3 was attained. Homologous HI readings in two weeks using Kaolin extracted sera yielded titers of 1:1280-1:2560. Heterologous HI titers were 1:320 against the WE antigen and 1:40 against the EE antigen. While the use of the Kaolin extraction was not consistently superior to the protamine sulfate or the sucrose acetone methods, it appeared consistently definitive when tested with VE, WE and EE viruses and was easier to use in the laboratory.

TABLE 2

COMPLEMENT FIXING (CF), HAEMAGGLUTINATION INHIBITION (HI) AND NEUTRALIZING (SN) ANTIBODY RESPONSES OF COYOTE NO. 4372 AFTER INOCULATION OF VENEZUELAN ENCEPHALITIS (VE) VIRUS*.

Weeks	CF (Thorpe)	NI (Lundgren)	Haemagglutination Inhibition Tests					
			D. L. Lundgren			R.F.N. Woo		
			VE Virus			VE	WE	EE
			Protamine Acetone	Kaolin	Sucrose Acetone	Kaolin	Kaolin	Kaolin
0	-	-	-	-	-	-	10	-
1	-	2.7	160	-	-	1280	20	20
2	64	3.3	640	1280	-	2560	320	40
3	64	4.6	1280	1280	1280	2560	640	40
4	16	3.7	640	1280	1280	5120	320	40
6	64	4.9	1280	1280	1280	640	160	80
7	-	5.1	-	-	-	640	-	10
21	16	4.7	1280	320	1280	1280	10	10
31	64	4.8	1280	640	1280	2560	10	40
41	64	4.7	1280	1280	1280	-	-	-

* Inoculum was $10^{5.7}$ VE Virus 38873, M2SM2 21 day old mouse IC LD_{50} doses given subcutaneously: Lundgren & Smart, 1969.

387

SMART

Figures 1 and 2 show the antibody responses of the seven coyotes held for 41 weeks after inoculation of VE viruses. Figure 1 shows the VE virus HI antibody responses to be rapid, reaching peak titers by one week after inoculation. The VE virus CF antibody was not detectable until the second week and peak titers were reached by the third week. Significant VE SN antibody levels were detectable by one week and reached peak neutralizing indices in three weeks. The same general range of antibody levels was maintained during the 41 weeks of observation. In Figure 2 it may be seen that HI antibody that cross-reacted with WE and EE antigens reached peak titers at three weeks as compared to one week for VE antibody. WE titers maintained their levels during the 41 week period while EE titers become negative. The need for a selected spectrum of antigens in an arbovirus survey is suggested from these observations.

Comparative VE Virus Infections in Native Rodents (12)

Groups of Peromyscus maniculatus (Pm), the Deer mouse, Reithrodontomys megalotis (Rm), the Harvest mouse, Onychomys leucogaster (O.L.), the Grasshopper mouse, P. truei (Pt), the Pinyon mouse and P. crinitis (Pc), the Canyon mouse and both young and adult white mice were inoculated subcutaneously with graded doses of VE virus. The virus suspension was from primary chick embryo cell culture infected with a thrice cloned VEE-SP (small plaque) virus derived from the Colombian strain 38873 M2SM1. Based upon plaque assay the animals received 6,600 and 60,000 PFU per inoculum dose. The data accumulated in 21 days are shown in Table 3. Surviving animals were bled from the orbital sinuses at 21 and 136 days after infection. Shown in Table 4 are HI and plaque neutralization tests performed on 136 day specimens.

Tables 3 and 4 support the following conclusions:

1. The thrice cloned mutant of VE virus used in these studies was of reduced virulence for the native rodents and both young and adult white mice.

2. Among the animals receiving an infective inoculum of 6 PFU and surviving to 136 days, only one, an O. leucogaster, exhibited an antibody response (HI 640 and significant evidence of N-pr antibody).

3. Based on the above information of an antibody response of an O. L. and deaths of individuals of all species and mice except the adults injected with the lowest dose of virus, this inoculum contained infective virus.

4. In the case of all animals receiving 600 or 60,000 PFU VE-SP virus that survived; all had neutralizing antibody at 21 days and all exhibited both HI and N antibody titers 136 days after

challenge.

5. Using described serological methods, all sera collected from animals 136 days post-challenge that showed a HI titer of ±320 or greater also possessed neutralizing antibody titers to VEE virus. These data, though few, strongly suggest that suspicion should be attached to the interpretation of HI titers of Kaolin-adsorbed rodent sera with VE HI titers of 1:80 and less.

The hypothesis is inherent that the VE virus strain used, VE-SP, is the prototype of strains which exist in nature under certain conditions and which are responsible for detectable antibody in the sera of animal species, highly susceptible to the lethal effects of wild "virulent" strains.

TABLE 3 (12)

MORTALITY RATIOS OF GROUPS OF NATIVE RODENTS AND LABORATORY MICE INFECTED WITH THE VENEZUELA ENCEPHALITIS VIRUS.

Species	PFU VE Virus 3887[2] M2SM1-ccp-3	Mortality Ratio 21 Day
Deer mouse	6	1/7
	600	6/7
	60000	7/7
Pinyon mouse	6	4/7
	600	6/6
	60000	7/7
Harvest mouse	6	2/7
	600	6/7
	60000	7/7
Grasshopper mouse	6	3/8
	600	7/8
	60000	8/8
Canyon mouse	6	2/2
	600	2/2
	60000	2/2
White mouse (adult)	6	0/5
	600	3/5
	60000	1/5
White mouse (young)	6	2/5
	600	4/5
	60000	2/5

Survivors were bled and held for 136 days.

389

SMART

TABLE 4 (12)

HAEMAGGLUTINATION INHIBITION AND PLAQUE NEUTRALIZING ANTIBODY TITERS OF SERA FROM NATIVE RODENTS AND LABORATORY MICE SURVIVING VENEZUELAN ENCEPHALITIS VIRUS INFECTION.

Animal Species	VE Virus 38873 Virus Dose M2SM1-ccp-3 as PFU	Serum Titer Plaque Reduction 21 Days	136 Days PR	136 Days HI
Deer mouse	6	TMC	TMC	20
	6	TMC	TMC	20
	6	TMC	TMC	80
	6	TMC	TMC	10
	6	TMC	TMC	20
	600	0	0	±1280
Pinyon mouse	6	TMC	TMC	10
	6	TMC	TMC	10
Harvest mouse	6	TMC	TMC	20
	6	TMC	TMC	20
	6	TMC	TMC	10
Grasshopper mouse	6	TMC	TMC	10
	6	TMC	TMC	10
	6	TMC	TMC	10
	6	TMC	TMC	10
	6	Broken	13	±640
White mouse (adult)	6	TMC	TMC	10
	6	TMC	TMC	10
	6	TMC	TMC	10
	600	TMC	TMC	10
	600	0	0	320
	60000	0	0	1280
	60000	0	0	1280
	60000	0	0	1280

Experimental Viral Interference Studies (13)

The problem of viral interference was studied to elucidate the nature of co-infection by two viruses and the nature of suppression of one infection by another as this related to the problems of interpretation of diagnostic and ecological infections data.

When suckling mice were simultaneously inoculated with a mixture of VE and WE viruses interference in the replication of WE virus was observed. (Table 5). The singly infected WE virus control mice died in 56, 48 and 44 hours respectively in suckling mouse brain passages 1, 2 and 3. The VE viral control mice died in 40, 20 and 20 hours respectively. The group of suckling mice receiving 100 PFU of WE virus and either 0.1 or 1.0 PFU of VE virus survived 50, 38 and 24 hours for the three mouse brain passages. Although these animals received a small dose of VE virus it was noted that the mice of the 2nd and 3rd passages died of VE virus infection. The mouse brain material for each dose combination and passage level was assayed by the plaque assay method to determine exact concentration of WE and VE viruses in each sample. Table 6 shows the virus titers obtained in singly infected VE and WE virus control animals. A one log difference was observed in the maximum titer of the two viruses.

TABLE 5 (13)

TIMES OF DEATH OF SUCKLING MICE INFECTED WITH VARYING COMBINATIONS OF WEE AND VEE VIRUSES

Infective Dose PFU/0.02 ml		Time of Death in Hours Post Infection		
WEE	VEE	SM1	SM2	SM3
10	0	56	48	44
0	10	40	20	20
100	0.1	50	38	24
100	1.0	50	38	24
100	10	40	20	20
100	100	40	20	20
0.1	100	40	20	20
1.0	100	40	20	20
10	100	40	24	20

TABLE 6 (13)

TITERS OF WEE AND VEE VIRUS CONTROLS IN SM1, SM2, AND SM3 JSE BRAIN PASSAGES INITIALLY INFECTED WITH 10 PFU/0.02 ML

Passage Level	WEE Virus PFU/ml	VEE Virus PFU/ml
SM1	1.92×10^8	
SM2	4.45×10^9	
SM3	7.90×10^9	
SM1		6.25×10^{10}
SM2		8.82×10^{10}
SM3		9.00×10^{10}

Titers of the individual viruses in many dose combinations and passage levels were tested; suffice it here to show the data on the interfering effect of VE virus on WE propagation in suckling mice initially infected with 100 PFU of WE virus and 10 PFU of VE virus. Table 7 shows that the normal concentrations of VE virus were produced throughout three mouse brain passages while the WE titer was reduced throughout the three passages by about two logs in each successive brain passage.

Upon infection of chick embryo cells with these two viruses interference of either virus by the other could be demonstrated by giving the interfering virus an advantage over the challenge virus. This advantage could be one of either time or concentration. Table 8 shows interference between WE and VE viruses in primary chick embryo cells infected simultaneously with equal multiplicity of infection (input PFU/cell). About 0.5 logs less WE virus than VE virus was produced with all concentrations used.

TABLE 7 (13)

TITERS OF WEE AND VEE VIRUSES IN MOUSE BRAIN PASSAGES INITIALLY INFECTED WITH 100 PFU/0.02 ML WEE VIRUS AND 10 PFU/0.02 ML VEE VIRUS

Passage Level	WEE Virus PFU/ml	VEE Virus PFU/ml
SM1	2.08×10^8	4.30×10^{10}
SM2	3.65×10^6	1.13×10^{11}
SM3	9.50×10^4	9.00×10^{10}

TABLE 8 (13)

INTERFERENCE BETWEEN WEE AND VEE VIRUSES IN PRIMARY CHICK EMBRYO CELLS INFECTED SIMULTANEOUSLY WITH EQUAL MOI*

MOI		WEE Virus	VEE Virus
WEE	VEE	PFU/ml	PFU/ml
5	0	4.50×10^9	
0	5		4.50×10^9
10	10	4.50×10^8	2.58×10^9
5	5	3.88×10^8	1.65×10^9
1	1	7.00×10^8	1.99×10^9
0.1	0.1	3.75×10^8	1.00×10^9

* Multiplicity of infection.

SMART

Table 9 shows the effect of input multiplicity of infection (MOI) on the interference between WE and VE viruses in chick embryo cells. It is seen that either virus was capable of interfering with the replication of the other if given a multiplicity advantage. The greater the difference of multiplicity of infection the greater was the difference in interference ratios.

TABLE 9 (13)

EFFECT OF INPUT MULTIPLICITY OF INFECTION ON THE INTERFERENCE BETWEEN WEE AND VEE VIRUSES IN CHICK EMBRYO CELLS.

MOI*		WEE Virus	VEE Virus
WEE	VEE	PFU/ml	PFU/ml
10	0	3.75×10^9	
0	10		5.50×10^9
100	0.1	3.69×10^9	$< 1.00 \times 10^7$**
100	1.0	2.25×10^9	1.63×10^8
100	10	1.63×10^9	6.75×10^8
100	100	4.88×10^9	3.20×10^9
0.1	100	$< 1.00 \times 10^7$	4.85×10^9
1.0	100	$< 1.00 \times 10^7$	3.00×10^9
10	100	4.50×10^7	2.22×10^9

* Multiplicity of infection
**Lowest limit detectable

When equal multiplicities of two viruses were used, the degree of interference was dependent upon the time period between infection by the first virus and superinfection by the challenge virus. Table 10 shows the interference of WE by VE virus. The data indicate that the degree of interference was increased as the time prior to superinfection was increased. Similar results for interference of VE virus by WE virus were obtained.

In similar studies infection of suckling mice with a combination of WE and CE viruses resulted in no interference between the two. Regardless of high multiplicity of infection advantage given either virus both were able to replicate in the presence of WE virus regardless of advantages in time or multiplicity of infection.

Similarly, interference of CE virus by VE virus was observed when the two viruses were inoculated simultaneously into suckling mice. CE virus was unable to interfere with VE virus even when given a multiplicity advantage. Studies in chick embryo cells yielded similar results to those obtained in suckling mice.

TABLE 10 (13)

EFFECT OF TIME OF SUPERINFECTION ON INTERFERENCE OF WEE VIRUS BY VEE VIRUS IN CHICK EMBRYO CELLS

Time of Infection by VEE Virus*	Time of Infection by WEE Virus*	VEE Virus PFU/ml	WEE Virus PFU/ml
0 Time	-	2.20×10^9	
-	0 Time		4.25×10^9
Control	1 Hr		3.85×10^9
0 Time	1 Hr	3.60×10^9	6.00×10^7
Control	3 Hrs		3.60×10^9
0 Time	3 Hrs	3.50×10^9	$< 1.00 \times 10^6$**
Control	5 Hrs		4.06×10^9
0 Time	5 Hrs	1.65×10^9	$< 1.00 \times 10^6$

* Multiplicity of infection = 5
** Lowest limit detectable

CONCLUSIONS

1. Comparative HI reactions on Kaolin absorbed sera from native rodents and rabbits indicate the unreliability of HI titers lower than 1:160 as an indicator of the presence of specific neutralizing antibody to the VE virus.

2. When tested in coyotes experimentally infected with VE virus, haemagglutination inhibition data are equally as good using either of three methods of serum extraction, i.e., Protamine Acetone, Kaolin and Sucrose Acetone. However, the serological cross reactivity with WE antigen observed, suggests that any titer less than 1:320 may not be definitive as an indicator of a significant level of neutralizing antibody.

3. Data from a 41 week study of serological responses of coyotes to experimental VE virus infection supports the conclusion that VE virus infection may be readily diagnosed by haemagglutination inhibiting, complement fixing and neutralizing antibody test methods between one and 41 weeks after exposure. The need for utilizing related cross reactive antigens along with the antigen of sentinel interest in conducting a serological survey is critical.

4. Pertinent to experimental infection studies on native mice a thrice cloned mutant of VE virus, No. 38873 (Colombia strain) M2SM1, was of reduced virulence such as to simulate a surviving

virus in nature. Its use in experimental infections permitted the observation that all surviving rodent species used developed neutralizing antibody by 21 days and all exhibited neutralizing and haemagglutination inhibiting antibody 136 days after challenge. These data indicated the presence of neutralizing antibody in sera with titers of ± 1:320 or greater.

5. Viral interference between two arboviruses in host systems of suckling mice or primary chick embryo cells yielded the following conclusions:

(a) Interference between WE and VE viruses in both suckling mice and primary chick embryo cells was reciprocal as long as an advantage of time or multiplicity was given one of the viruses; (b) no interference between WE and CE viruses was observed in suckling mice, however, WE virus readily interfered with the replication of CE virus in chick embryo cells; (c) interference of CE virus by VE virus was demonstrated in both suckling mice and chick embryo cells; California virus was incapable of interfering with either WE or VE virus.

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397

SMART

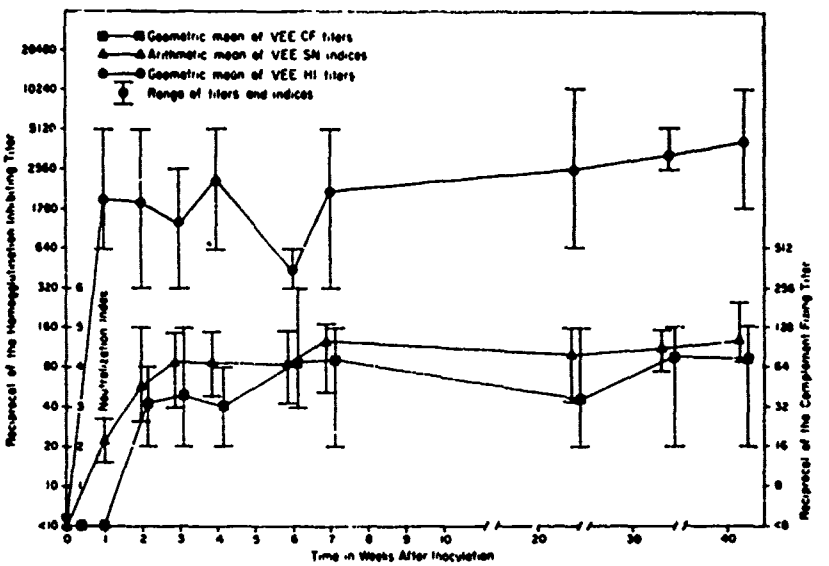

Figure 1 Complement fixing (CF), hemagglutination inhibiting (HI), and neutralizing (SN) antibody responses of seven coyote pups after the inoculation of Venezuelan equine encephalitis (VEE) virus

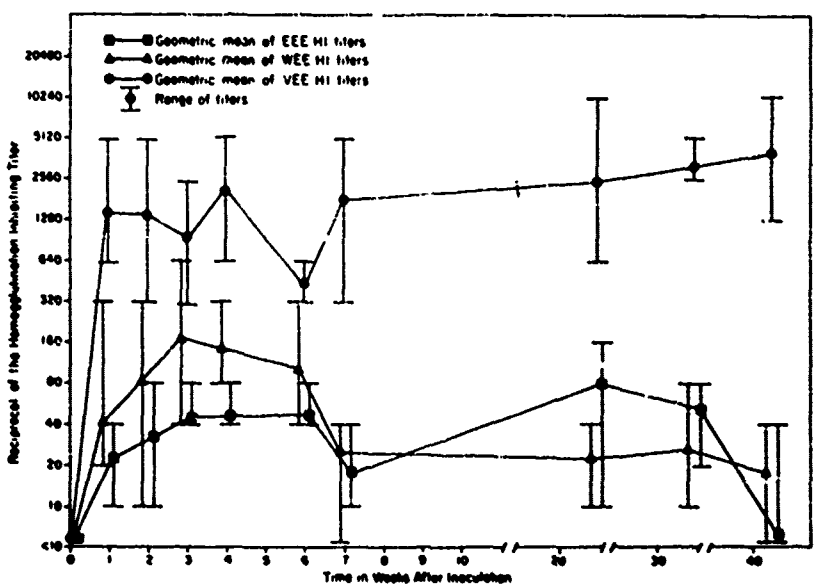

Figure 2 Hemagglutination inhibition (HI) of Venezuelan equine encephalitis (VEE), Western equine encephalitis (WEE) and Eastern equine encephalitis (EEE) viruses by the sera of seven coyotes inoculated with VEE virus

359 Exhibit